Three-dimensional human skin wound infection equivalent as a tool for bioanalysis of antimicrobial polymeric biomaterials for wound dressings

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Introduction: The aim of this study was the development of a human skin wound infection model for the bio-evaluation of antimicrobial biomaterials intended for wound healing purposes. Development of human-based three-dimensional *in vitro* systems with bacterial infection and biofilm formation will serve as an advanced and complex system to perform more reliable preclinical studies. These systems will be employed for *in vitro* screening of both antibacterial activity as well as cytocompatibility of the studied material to obtain better *in vivo* performances.

Methods: The 3D skin equivalent was obtained having both a dermal and an epidermal compartment, by embedding human primary fibroblasts in rat tail tendon collagen type I hydrogel (mimicking skin extracellular matrix) and then seeding human primary keratinocytes on it to generate the epidermal layer. The model was characterized for morphological characteristics and dermal/epidermal markers through histological and immunohistochemical analysis, respectively. To assess the viability of the system quantitatively, different cell viability and cytotoxicity assays e.g. CellTiter-Blue®, CytoTox-ONE™, RealTime-Glo™ MT, CellTiter-Glo® (Promega) were evaluated to finally optimize the best suited assay to the respective cell types and eventually to the 3D system.
This model would be inoculated with clinically challenging bacteria e.g. Staphylococcus aureus to generate a 3D wound infection model.

Visualization of dermal fibroblasts in 3D

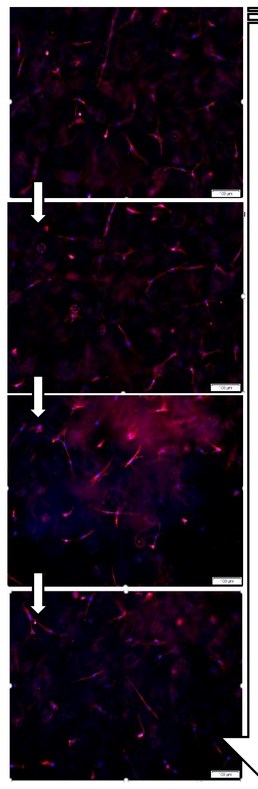


Fig 1: The model was established by first optimizing the dermal part of skin. The Z-stacked imaging revealed the filopodia like morphology and a uniform distribution of human fibroblasts at different planes inside the matrix. The dermal layer showed fibroblasts with dendritic extensions in the matrix. However, no morphological differences were found among different collagen I matrix concentrations. Fluorescent microscopic images show cell nuclei stained with DAPI and F-actin of cells stained with Phalloidin. Scale bar=100 µm

Development of 3D skin model

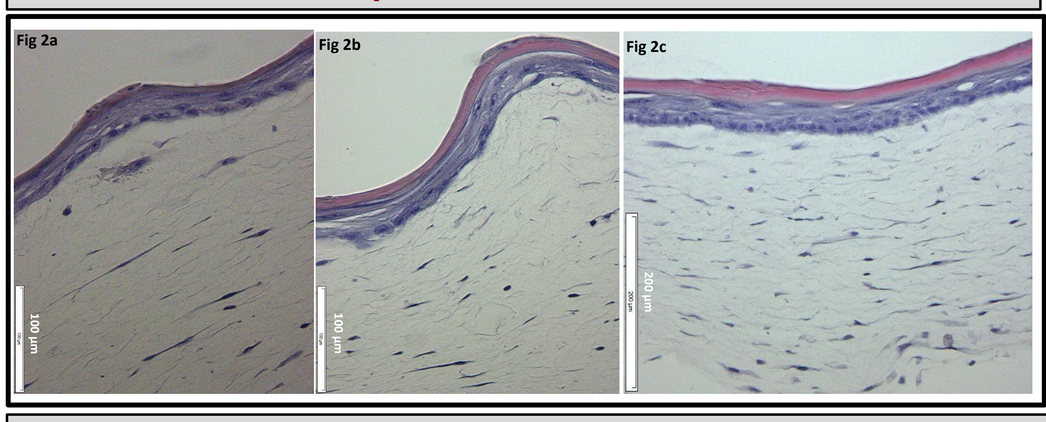


Fig 2: Hematoxylin/Eosin (H & E) stained histological images of *in vitro* human skin model after 10 days at Air Liquid Interface (ALI) (a), 15 days at ALI (b), 20 days at ALI (c). The results showed the two structurally distinct layers of skin: the outer epidermis layer and the underlying thicker dermis layer that consists of connective tissue. The epidermal part showed well differentiated layers of keratinocytes namely stratum corneum, granulosum, spinosum and basale, well-organized after 20 days at ALI. Furthermore, the contact between dermal and epidermal surface was not straight rather it was undulating in a way that it intertwined with dermal layer; mimicking the *in vivo* situation. Scale bar= 100 and 200 µm

Immunohistostaining

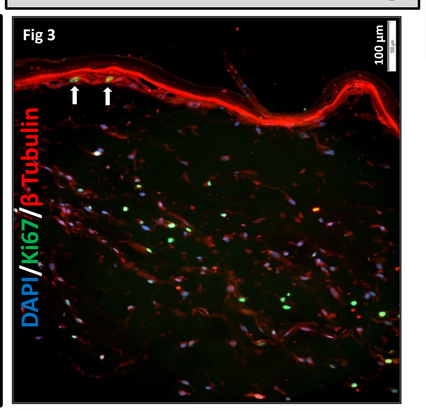


Fig 3: Immunolabelling for specific epidermal marker, Ki67 (arrows) showed the proliferative state of basal epidermal cells, demonstrating the analogies in the differentiation program with *in vivo* situation. Scale bar=100 µm

Quantitative measurement of cell viability of 3D fibroblasts (in a preliminary dermal model)

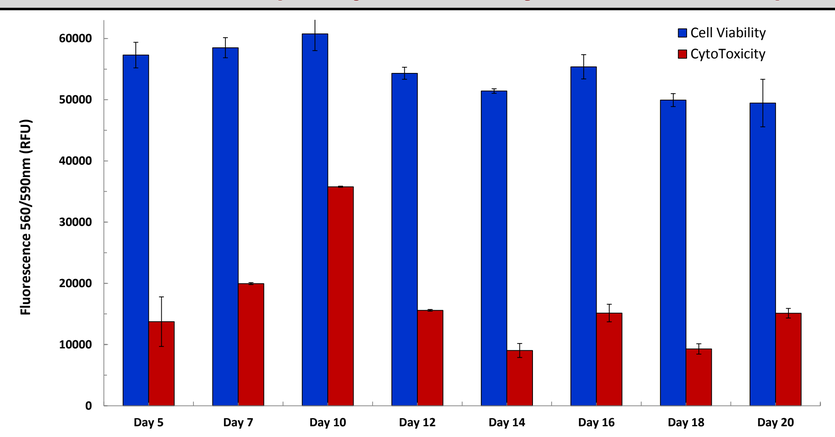
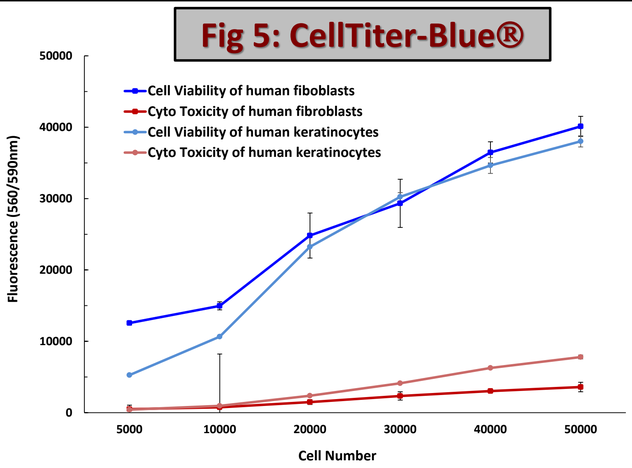


Fig 4: A dermal model based on L929 fibroblasts was used for optimization of cytocompatibility measurement as a quantitative analysis of viable and dead cells overtime. The results showed that cells stayed viable inside the matrix for at least 20 days that is a pre-requirement of the *in vitro* model. Both assays (CellTiter-Blue® and CytoTox-ONE™) were adapted and optimized to this 3D dermal system. Repeated exposure of cells to reagent (results not shown here) up to 18 days had no lasting toxic effect. Therefore, this assay is suitable as a method to monitor cell viability of the same sample over an extended time frame.

Optimization of cell viability measurement of human fibroblasts

Fig 5: CellTiter-Blue®



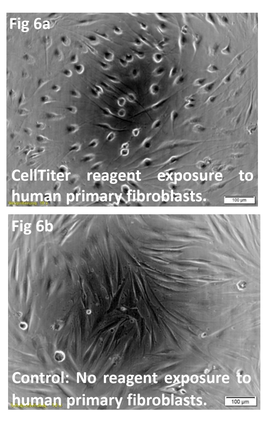


Fig 5: The results showed that CellTiter-Blue® assay was able to measure cell viability of primary cells (human fibroblasts and keratinocytes). However, microscopic observations (**Fig 6a,b**) showed that reagent affected cell morphology of human fibroblasts (unlike L929 fibroblast) indicating reagent interference with cell normal biological activity, resulting in less reliable data. Therefore, this assay would not provide definitive results in a clinically relevant model based on human primary cells (that is more sensitive than a model based on cell lines). Scale bar=100 µm

Fig 7: CellTiter-Glo®

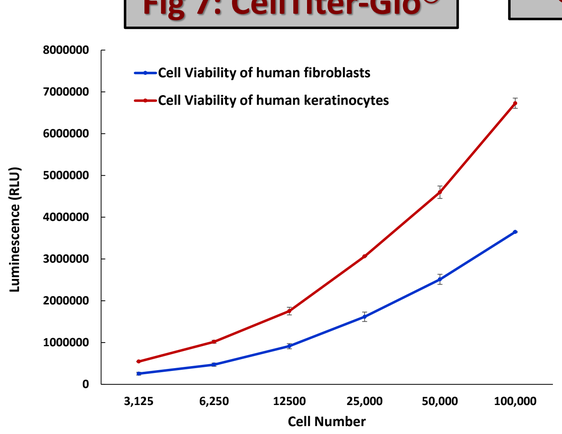


Fig 7: CellTiter-Glo® was found to be the optimal cell viability assay among those analyzed. For example, CellTiter-Blue® affected cell morphology and RealTime-Glo™ did not provide a linear signal with even as low as 2500 cell number (data not shown), which made these assays unsuitable for the system.
Results showed that the CellTiter-Glo assay was able to measure as high as 100,000 cells.
Parameters were optimized for this assay (data not shown). For example, results showed that half life of signal measured was more than 4 hours.

Development and optimization of quantitative cytocompatibility assays for *in vitro* human skin model

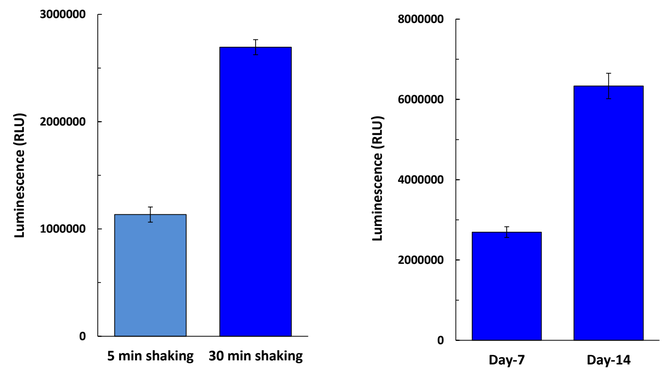


Fig 8: The graphs show the results of viability of human primary fibroblasts in 3D dermal model. Therefore, after selecting CellTiter-Glo® as an appropriate assay, cell viability in the 3D dermal model (based on primary dermal fibroblasts) was measured over time and the results showed that cells not only stayed viable inside the matrix but were also proliferating over time.
Protocol was improved for this assay in 3D system by optimizing the shaking time. Results showed that increased shaking time resulted in higher cell lytic capacity for maximum ATP release. 30 minutes shaking time for a "7 days cultured dermal model" gave 2.4 times higher value than the one at lower shaking time of 5 min.
However, shaking times varied for the measurement of cell viability of 3D system at different time points, indicating the matrix-cell interaction and thus, density variation with increasing culture time.

Testing of medical biomaterials

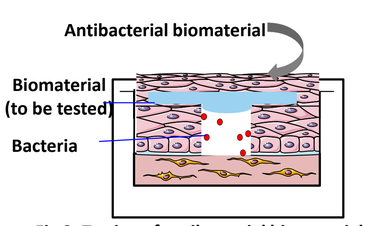


Fig 9: Testing of antibacterial biomaterials intended for wound healing purposes

Fig 9: Future perspective: Staphylococcus aureus, a major bacterial pathogen would be allowed to grow in this 3D model to mimic skin infection model. This would serve as an *in vitro* tool recapitulating enough biological response for the bio-evaluation of antimicrobial and wound healing properties of novel anti microbial biomaterials.

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